

Identification of a novel compound heterozygous *CYP4V2* variant in a patient with autosomal recessive retinitis pigmentosa

TONGDAN ZOU^{1*}, TING WANG^{1*}, FANGYUAN ZHEN², SHUQIAN DONG²,
BO GONG^{1,3} and HOUBIN ZHANG^{1,3}

¹The Key Laboratory for Human Disease Gene Study of Sichuan Province and Institute of Laboratory Medicine, Sichuan Provincial People's Hospital, University of Electronic Science and Technology of China, Chengdu, Sichuan 610072; ²Department of Ophthalmology, The First Affiliated Hospital of Zhengzhou University, Henan Provincial Ophthalmic Hospital, Zhengzhou, Henan 450000; ³Research Unit for Blindness Prevention of Chinese Academy of Medical Sciences, Sichuan Academy of Medical Sciences and Sichuan Provincial People's Hospital, Chengdu, Sichuan 610072, P.R. China

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Abstract. Retinitis pigmentosa (RP) belongs to a family of retinal disorders that is characterized by the progressive degeneration of rod and cone photoreceptors. The aim of the present study was to screen for possible disease-causing genetic variants in a non-consanguineous Chinese family with non-syndromic autosomal recessive RP. Whole-exome sequencing (WES) was performed in samples from the affected individual (the proband) and those from the two children of the proband. A novel compound heterozygous variant of c.C958T (p.R320X) and c.G1355A (p.R452H) in the *Cytochrome P450 family 4 subfamily V member 2 (CYP4V2)* gene was identified through WES. Subsequently, this variant was validated by direct Sanger sequencing. This compound heterozygous variant was found to be absent from other unaffected family members and 400 ethnically-matched healthy control individuals. In addition, this compound variant was co-segregated with the RP phenotype in an autosomal recessive manner. *In silico* analysis revealed that both c.C958T (p.R320X) and c.G1355A (p.R452H) could compromise the protein function of CYP4V2. These results strongly suggest this compound variant to be a disease-causing variant, which expands upon the spectrum of currently known *CYP4V2* genetic variants associated with retinal diseases.

Introduction

Retinitis pigmentosa (RP; OMIM no. 268000) is a group of highly heterogeneous but related retinal disorders that cause progressive vision loss (1-3). Typically, RP manifests as night blindness in the early stages. As this disease progresses, the extent of visual field loss becomes gradually more apparent, with impaired color vision and fundus degeneration during the advanced stages. The prevalence of RP is ~1/4000 in China (4,5). RP can be classified as syndromic or non-syndromic. Usher syndrome and Bardet-Biedl syndrome, which also affect multiple organs, are the most common forms of syndromic RP. By contrast, non-syndromic RP is typically inherited and can manifest in an autosomal-recessive (50-60% of cases), autosomal-dominant (30-40% of cases), X-linked (5-15% of cases) or mitochondrial manner (6-8). In the pathophysiology of all types of RP, the majority of mutant genes reported are expressed exclusively in rod cells. Although a small number of mutants are specifically expressed in the retinal pigment epithelium, none are cone-specific (<https://sph.uth.edu/retnet/>). Despite this, RP can cause the degeneration of both rod and cone photoreceptors, which mediate achromatic night vision and high acuity central vision, respectively (9-14).

One of the reasons for the heterogeneity of RP is the >80 disease-causing genes that have been identified (<https://sph.uth.edu/retnet/>). Additionally, variations in these genes can cause a wide range of clinical symptoms that are distinct from typical RP, including cone-rod dystrophy (CORD), Leber's congenital amaurosis (LCA) and stationary night blindness. For example, whilst a number of variants in the *cone-rod homeobox (CRX)* gene have been reported to be associated with RP, other variants of *CRX* can also trigger CORD and LCA (15-18). In another example, although the majority of *Cytochrome P450 family 4 subfamily V member 2 (CYP4V2)* variants are associated with Bietti crystalline dystrophy (BCD), other variants in the gene can also cause RP. BCD is an autosomal recessive chorioretinal degenerative disease that is characterized by numerous glistening yellow-white crystalline retinal micro-deposits, progressive atrophy of the retinal pigment epithelium (RPE) and choroidal sclerosis (19).

Correspondence to: Dr Houbin Zhang or Dr Bo Gong, The Key Laboratory for Human Disease Gene Study of Sichuan Province and Institute of Laboratory Medicine, Sichuan Provincial People's Hospital, University of Electronic Science and Technology of China, 32 The First Ring Road West 2, Chengdu, Sichuan 610072, P.R. China

E-mail: houbin_zhang@yahoo.com

E-mail: gongbo2007@hotmail.com

*Contributed equally

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In the present study, whole-exome sequencing (WES) was applied to screen for potential disease-causing variants in a non-consanguineous Chinese family with autosomal recessive RP. A novel compound heterozygous variant in *CYP4V2* was identified in a patient with RP.

Materials and methods

Subjects. A Chinese family with RP, including six members with an affected individual and five unaffected individuals, was recruited from the Sichuan Provincial People's Hospital (Chengdu, China). The affected individual (sex, female) was 47 years old when diagnosed. Additionally, 400 unrelated healthy subjects, including 218 males and 182 females, were recruited from the Sichuan Provincial People's Hospital (Chengdu, China). The median age of the healthy controls was 42 (age range, 20–60). The study was performed in accordance with the tenets of the Declaration of Helsinki (20) and approved by the Institutional Review Boards of Sichuan Academy of Medical Sciences and Sichuan Provincial People's Hospital. Signed informed consent was obtained from all participants before their inclusion in this study.

Clinical diagnosis. All participants underwent ophthalmological examinations. Fundus photography was performed for all members of the affected individual's family. The clinical data were assessed by ophthalmologists at Sichuan Academy of Medical Sciences and Sichuan Provincial People's Hospital.

DNA isolation. Peripheral blood samples were collected in EDTA tubes from all six members of the family and unrelated normal controls. Genomic DNA was extracted using a blood DNA extraction kit according to the manufacturer manual (Tiangen Biotech, Co., Ltd.) DNA samples were stored at -20°C until required.

Whole-exome sequencing (WES). The DNA from individuals II-1 (the proband), III-1 and III-2 were analyzed by WES with a mean read depth of 100x. The samples were prepared following the Illumina standard procedure (Illumina, Inc.). Briefly, $\sim 3\ \mu\text{g}$ genomic DNA was randomly sheared into small fragments of 150–220 bp using a sonicator (Covaris). The sheared fragments were purified with reagents supplied with the AMPure XP system (Beckman Coulter, Inc.). Adapters (Agilent Technologies, Inc.) were ligated with the polished ends and the libraries were amplified by PCR. The amplified libraries were hybridized with biotin-labeled probes in the liquid phase. The DNA fragments bound to the probes, namely the captured library, were purified. Then, the library was sequenced on a Illumina HiSeq4000 platform (Illumina, Inc.) and paired-end 150 bp reads were obtained.

Mutation identification and data analysis. The mutations in *CYP4V2* were identified using the following databases: dbSNP138 (<https://www.ncbi.nlm.nih.gov/snp/>), 1000Genomes Project (http://grch37.ensembl.org/Homo_sapiens/Info/Index), Exome Aggregation Consortium (<https://gnomad.broadinstitute.org/>), OMIM (<https://www.omim.org/>), and HGMD (<http://www.hgmd.cf.ac.uk/ac/index.php>), as well as an east Asian population databases (<https://blog.nus.edu.sg/sshsphphg/singapore-genome-variation/>), ftp://ftp.1000genomes.ebi.ac.uk/vol1/ftp/release/20130502/supporting/GRCh38_positions/, and the Retnet database (<https://sph.uth.edu/Retnet/>).

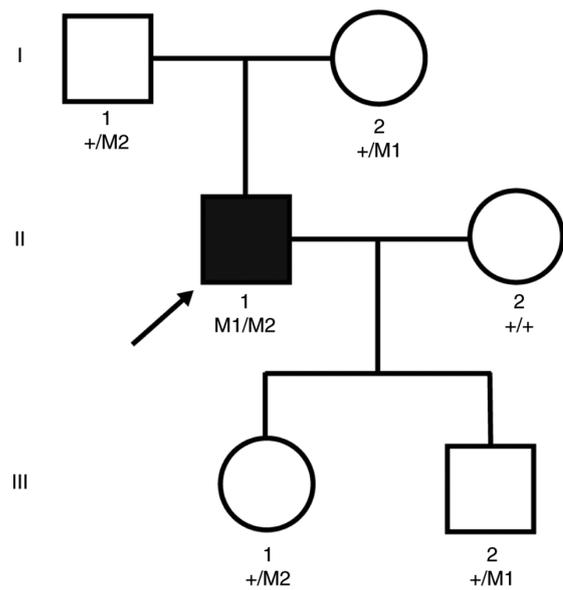


Figure 1. Pedigree of the Chinese family with retinitis pigmentosa. The arrow points to the proband. Females and males are indicated by circles and squares, respectively. Empty and filled symbols represent normal and affected individuals, respectively. M1 and M2 represent the genetic variants of c.C958T (p.R320X) and c.G1355A (p.R452H) in the *Cytochrome P450 family 4 subfamily V member 2* gene, respectively.

Sanger sequencing was used to verify the identified variants of *CYP4V2*. All exons of *CYP4V2* were amplified from the genomic DNAs by PCR under standard conditions (94°C for 2 min; followed by 30 cycles of 94°C for 20 sec, 58°C for 30 sec, and 72°C for 60 sec; with a final extension step of 72°C for 7 min) followed by sequencing on a 3730 ABI DNA sequencer (Thermo Fisher Scientific, Inc.). Finally, the sequencing results were analyzed using A plasmid Editor (version 2.0, by M. Wayne Davis at the University of Utah, USA). Online bioinformatics tools, including Mutation Taster (<https://www.mutationtaster.org/>), CADD (<https://cadd.gs.washington.edu/>), PROVEAN (<http://provean.jvvi.org/index.php>), PolyPhen-2 (<http://genetics.bwh.harvard.edu/pph2/>) (21), Panther (<http://www.pantherdb.org/tools/>) and Sorting Intolerant from Tolerant (SIFT) (<https://sift.bii.a-star.edu.sg/>), were used to predict the potential pathogenic effects of the amino acid substitution in *CYP4V2*. The mutant *CYP4V2* protein structures were generated by using SWISS-MODEL (<https://swissmodel.expasy.org/>).

Results

Clinical characteristics. A Chinese family consisting of three generations of individuals with RP, but no history of consanguineous marriage was examined in the present study (Fig. 1). Pedigree analysis suggested a pattern of autosomal recessive inheritance in this family, which consisted of a member with this disease and five who were unaffected. Fundus examination revealed that the proband exhibited the characteristic pathophysiology of RP (22), including retinal pigmented epithelium atrophy, attenuated blood vessels, retinal vascular attenuation and a waxy pallor optic disc. By contrast, fundus photography revealed no abnormalities in the unaffected individuals (Fig. 2).

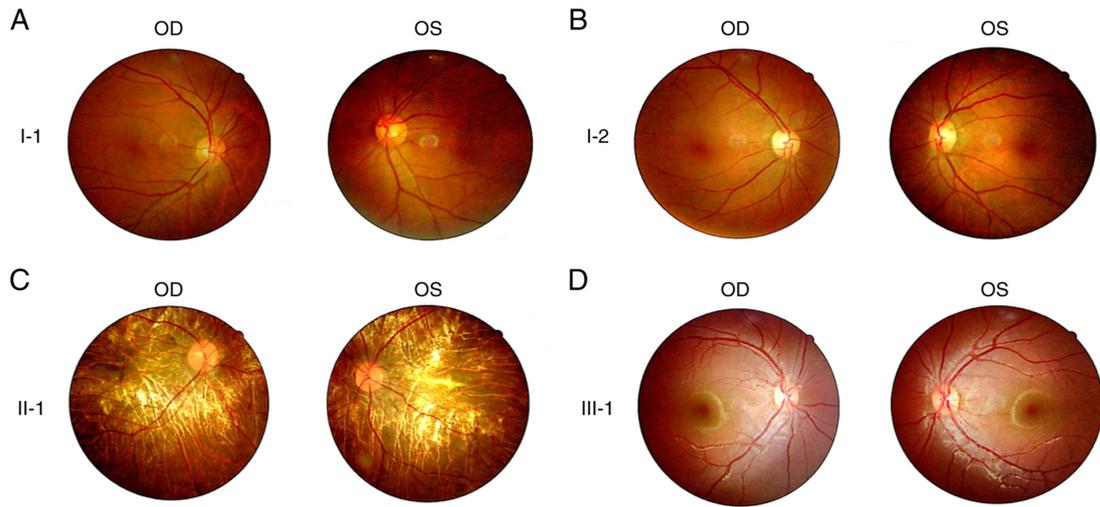


Figure 2. Fundus photographs. Fundus images of unaffected individuals (A) I-1, (B) I-2 and (D) III-1. (C) Fundus images of the affected individual (II-1) in the family. OD, right eye; OS, left eye.

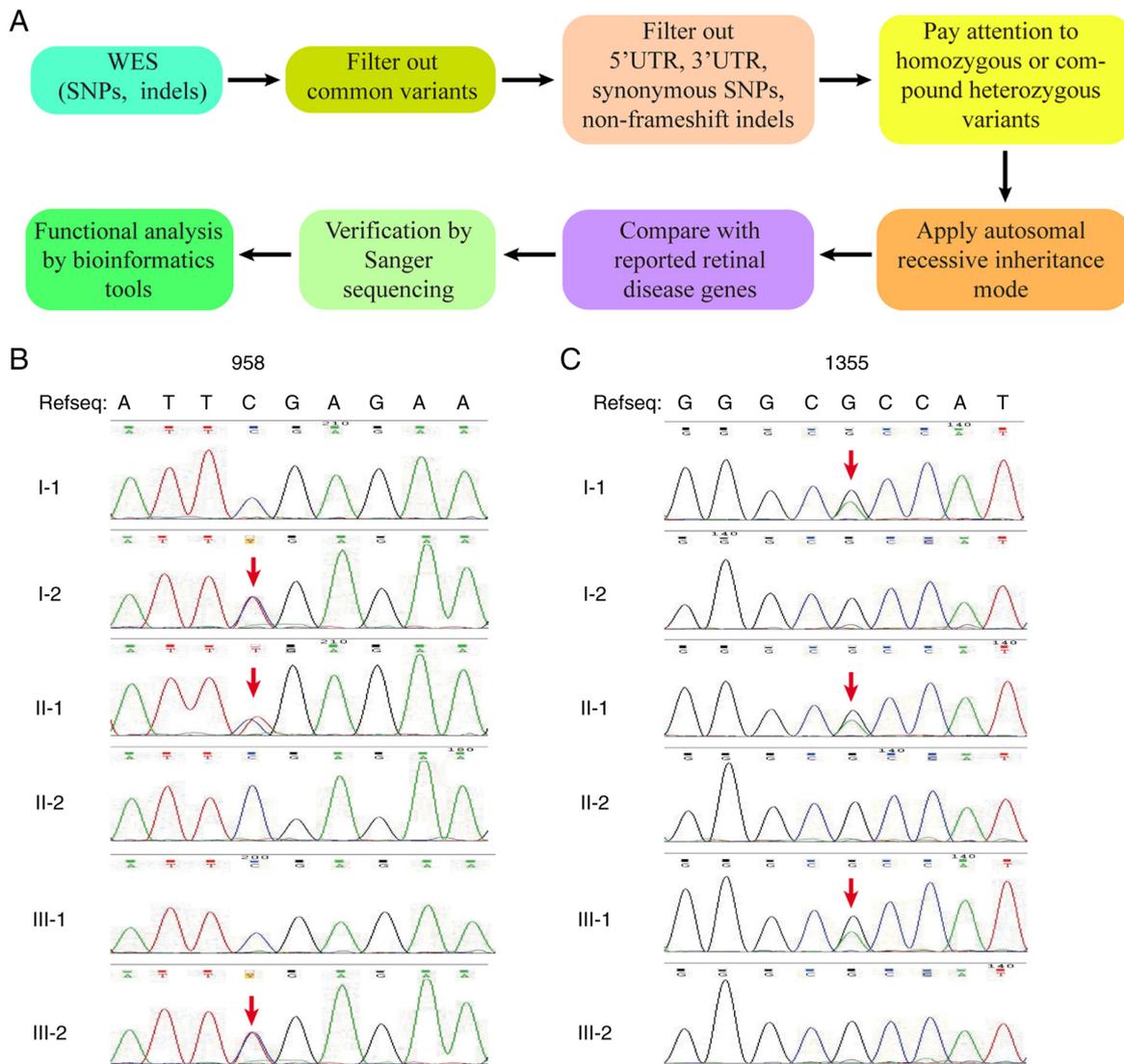


Figure 3. Identification of the mutations in the *CYP4V2* gene and validation using direct Sanger sequencing. (A) Flowchart showing the strategy for variant screening. (B) Partial sequence of exon 7 of the *CYP4V2* gene from all family members. The arrows indicate the site of the c.C958T (p.R320X) variant. (C) Partial sequence of exon 10 of the *CYP4V2* gene from all family members. The arrows indicate the site of the c.G1355A (p.R452H) variant. WES, whole exome sequencing; UTR, untranslated region; SNP, single-nucleotide polymorphism; *CYP4V2*, *Cytochrome P450 family 4 subfamily V member 2*; Refseq, reference sequence.

Table I. Bioinformatics analysis of the effect of p.R320X and p.R452H in CYP4V2.

CYP4V2 Mutation	Mutation Taster	CADD, RawScore /Phred	PROVEAN	PolyPhen-2	SIFT
p.R320X	Disease causing	8.551470/43, Deleterious	-13.84, Deleterious	N/A	N/A
p.R452H	Disease causing	4.218231/29.6, Deleterious	-4.79, Deleterious	0.999, Probably damaging	Damaging

CYP4V2, Cytochrome P450 family 4 subfamily V member 2; CADD, Combined Annotation-Dependent Depletion; PROVEAN, Protein Variation Effect Analyzer; SIFT, Sorting Intolerant from Tolerant.

WES and data analysis. After WES analysis on the proband (II-1), and individuals III-1 and III-2, 29,734 variants in the coding regions and splice junctions were obtained, including 14,298 nonsynonymous single-nucleotide polymorphisms (SNPs), 14,927 synonymous SNPs, 509 SNPs of miscellaneous types and 744 indels. To screen for the disease-causative variant in the family with RP, common variants present in high frequencies in dbSNP138, 1000 Genomes Project, Exome Aggregation Consortium, OMIM, HGMD and other east Asian population databases were filtered out. Subsequently, variants located in introns, 5'untranslated regions (UTRs) and 3'UTRs, in addition to synonymous SNPs and non-frameshift indels were also filtered out since they typically do not influence gene function. A particular focus was placed on possible functional SNPs/indels in the homozygous or compound heterozygous variants, including frameshift indels, non-synonymous variants and splicing junction variants, which are more likely to be pathogenic. These SNP/indels were filtered further using the criterion that the candidate variants must be inherited in an autosomal recessive inheritance manner. Genes affected by these filtered variants were then compared with the reported genes that have been previously associated with retinal diseases using the Retnet database (<https://sph.uth.edu/Retnet/>; Fig. 3A). As a result, a novel compound heterozygous variant, c.C958T (p.R320X) and c.G1355A (p.R452H), was identified in the CYP4V2 gene of the proband, but not in the other two unaffected family members. The segregation pattern of this compound variant was consistent with the clinical phenotypic and genetic profile of the family, suggesting that this is a candidate disease-causing variant.

Verification of variants in the CYP4V2 gene. Direct Sanger sequencing confirmed the compound heterozygous variant in the proband. The proband's father (I-1) and daughter (III-1) possessed a heterozygous variant of c.G1355A (p.R452H), his mother (I-2) and son (III-2) were heterozygous carriers of c.C958T (p.R320X) (Fig. 3B and C). The proband's wife (II-2) had no variant at either of these two positions. In addition, neither of the heterozygous compound mutations, homozygous c.G1355A (p.R452H) nor homozygous c.C958T (p.R320X) mutations could be detected in 400 ethnically-matched control samples. However, a heterozygous c.C958T variant was found in 2 of the 400 controls, whereas a heterozygous c.G1355A variant was found in 3 of the 400 controls. This compound heterozygous variant matched the genotype and phenotype of this family. These findings suggest that the RP symptoms present in the proband can be attributed to this compound variant in the CYP4V2 gene.

In silico analysis of the variants identified in CYP4V2. The c.C958T replacement caused the substitution of arginine with a stop codon (p.R320X), whilst the c.G1355A replacement caused the substitution of histidine with arginine (p.R452H). Amino acid sequence alignment of the CYP4V2 protein among different species revealed that these two variants are located in two highly conserved regions (Fig. 4A). Bioinformatics tools Mutation Taster, CADD, PROVEAN, PolyPhen-2 and SIFT, were used to analyze the potential impact of p.R452H on the function of CYP4V2. The results showed that these two variants are potentially pathogenic (Table I). The p.R320X mutation is located near the center of the P450 domain. The premature stop codon at this position results in the loss of the C-terminal portion of the protein, which accounts for ~33% of the protein. A model of the CYP4V2 protein structure generated using SWISS-MODEL showed that both mutants could greatly alter the structure of CYP4V2, which may affect its function (Fig. 4B-E).

Discussion

In the present study, a novel compound heterozygous CYP4V2 variant was discovered in a Chinese family with RP. To date, 129 variants of CYP4V2 have been reported to be associated with either BCD, corneal dystrophy, fundus dystrophy or RP (23-25). These variants include 82 missense mutations, 20 splicing substitutions, 17 small deletions, 4 small insertions, 3 small indels and 3 cross deletions (20-22).

Mutations in the CYP4V2 gene are documented to primarily result in BCD (26). BCD was first described in 1937 by Gian Battista Bietti. The typical clinical features of BCD include numerous yellow-white glistening crystalline deposits, progressive night blindness and constriction of the visual field (27). In the present study, a novel compound heterozygous CYP4V2 variant that could cause RP and not BCD was discovered in a Chinese family. The association of CYP4V2 with RP is not new, as this has been reported previously (23). However, RP is a highly heterogeneous retinal disease and it is not uncommon that mutations in the same gene can result in a variety of clinical manifestations. For example, mutations in RP GTPase regulator have been reported to cause either RP or cone-dystrophies in different individuals (28). In addition, RPE65 mutations can result in LCA and early-onset severe retinal dystrophy (29,30) or relatively mild phenotypes with slower rates of progression (29,31). One reason for this is that different mutations can mediate differential impacts on subsequent gene functions. In addition, another reason may be related to the different genetic backgrounds in different individuals, who can harbor different genetic modifiers.

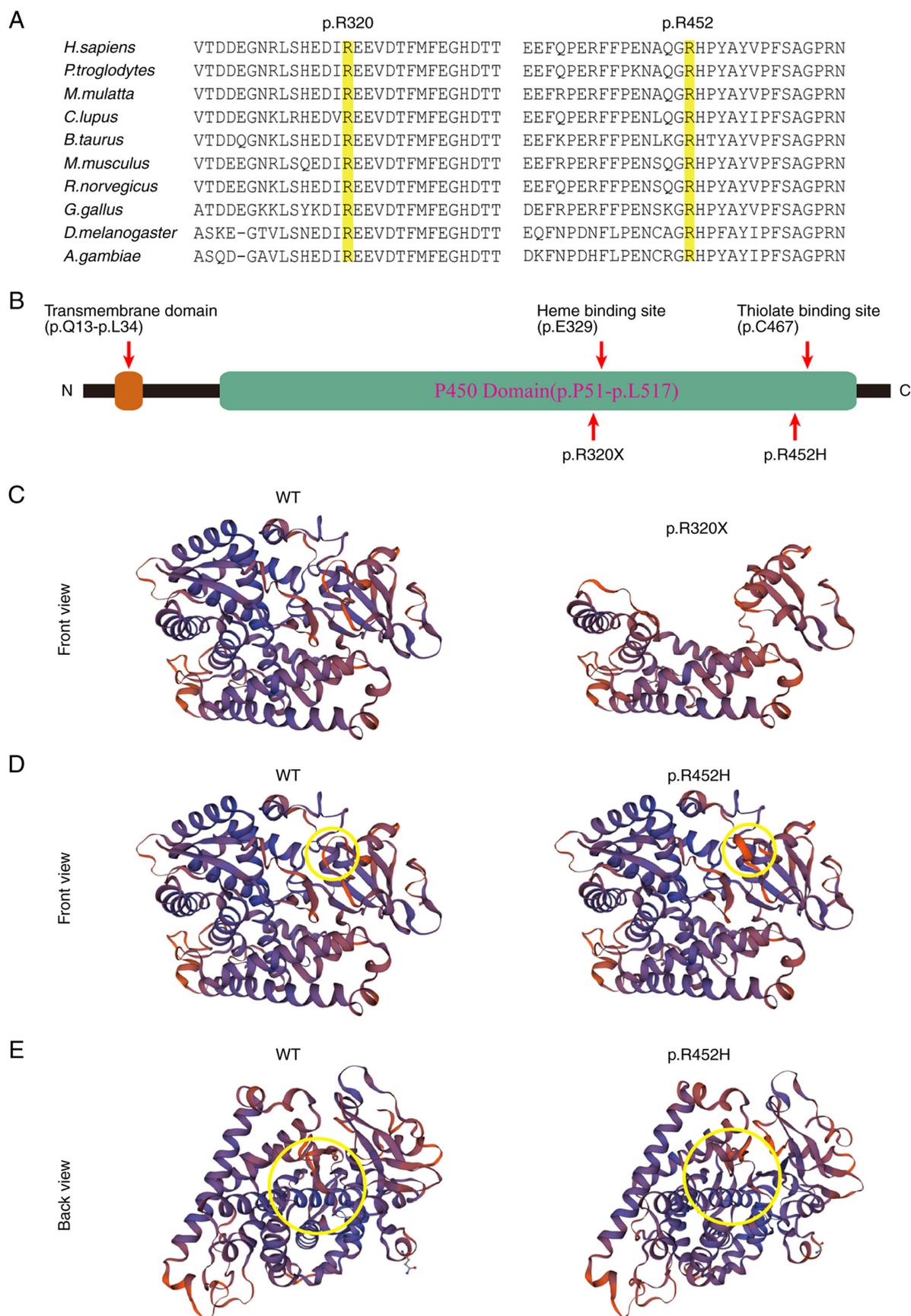


Figure 4. *In silico* sequence and structural analysis. (A) Sequence alignment of orthologous CYP4V2 proteins from 10 species. Conserved amino acids at positions 320 and 452 are highlighted in yellow. (B) Schematic showing the domain structure of CYP4V2. The heme-binding site at p.E329 and the thiolate-binding site at p.C467, as well as the positions of p.R320X and p.R452H mutations, are indicated by arrows. (C-E) Structures of WT, p.R320X mutant (c.C958T, p.R320X) and M2 p.R452H mutant CYP4V2 as modeled using SWISS-MODEL. (C) Front view of the WT and p.R320X mutant CYP4V2 structures. (D) Front view of the WT and p.R452H mutant CYP4V2 structures. (E) Back view of the WT and p.R452H mutant CYP4V2 structures. Yellow circles highlight the structural difference between WT and mutant CYP4V2. CYP4V2, Cytochrome P450 family 4 subfamily V member 2; WT, wild-type.

For example, a genetic modifier has been previously identified in patients with Bardet-Biedl syndrome (BBS) (32). A genetic variation in the *coiled-coil domain-containing 28B* gene, which encodes a pericentriolar protein, was found to greatly influence the phenotype of patients with BBS. Therefore, for any given disease, the symptoms exhibited are most likely to be the outcomes of interactions amongst multiple genes. As such, the precise phenotype in each individual may depend on the nature of mutations and the genetic modifier profile.

In the present pedigree, the proband (II-1) was found to be carrying the compound heterozygous variant of c.C958T and c.G1355A, whereas other family members were found to either carry none of the variants of interest or one heterozygous variant of c.C958T and c.G1355A. Furthermore, this compound heterozygous variant could not be found in 400 unrelated healthy Chinese control individuals or in any of the public databases probed, including HGMD (<http://www.hgmd.org/>), 1000 Genome project (<http://www.internationalgenome.org/>) and the NHLBI Exome Sequencing Project (ESP) Exome Variant Server (<http://evs.gs.washington.edu/EVS/>). Both c.C958T and c.G1355A in the *CYP4V2* gene were predicted to damage the function of the CYP4V2 protein according to PolyPhen-2. Therefore, the c.C958T and c.G1355A variants are likely to be putative pathogenic mutations. The homozygous c.C958T (p.R320X) or c.G1355A (p.R452H) mutation in the *CYP4V2* gene has been previously associated with BCD, supporting the notion that this compound heterozygous c.C958T (p.R320X) and c.G1355A (p.R452H) variant can cause retinal diseases (24,33,34).

CYP4V2 is also known as BCD or CYP4AH1, and belongs to a subfamily within the cytochrome P450 superfamily. *CYP4V2* is located on chromosome 4q35 and contains 11 exons, such that the CYP4V2 protein is ubiquitously expressed. In the eye, it is predominantly expressed in RPE cells with lower expression levels in the cornea. CYP4V2 is one of 57 functional human enzymes in the cytochrome P450 superfamily of heme-containing monooxygenase enzymes (35). Specifically, the CYP4V2 protein catalyzes the omega-3 hydroxylation of poly-unsaturated fatty acids (PUFAs), such as eicosapentaenoic acid and docosahexaenoic acid (36). PUFAs are widely distributed throughout the retina and are essential components of retinal rod outer segment membranes (37).

Structurally, *CYP4V2* encodes a protein containing 525 amino acid residues. As a member of the cytochrome P450 family, the CYP4V2 protein requires a cysteine thiolate-coordinated Fe(II) heme complex to activate the bound molecular oxygen in proximity to the cysteine thiolate (38). Therefore, the heme-binding and thiolate ligand-binding sites are indispensable for CYP4V2 function. The p.R320X mutant causes the premature termination of the polypeptide and truncation of ~33% of the protein at the C-terminus, which includes the heme-binding site (at E329) and thiolate ligand-binding site (at C467). This may severely impair enzymatic activity. Therefore, the p.R320X mutation was considered to be in the loss-of-function category. The p.R452H mutant is a missense mutation occurring at a position close to the thiolate ligand-binding site within the conserved P450 domain. The p.R452H mutant was predicted to alter protein conformation drastically, which may disrupt the formation of the cysteine thiolate-Fe(III) heme complex, thereby compromising the enzymatic function of CYP4V2. As a result, p.R452H was considered to be either a loss-of-function mutation

or a hypomorphic mutation. To conclude, this compound variant may severely impair the activity of CYP4V2, which is required for normal retinal function.

This compound variant of *CYP2V4* in this family described in the present study is different from one that was previously reported (c.802-8_810del17insGC) (23). The variants of c.802-8_810del17insGC and c.1091-2A>G were found to disrupt the splicing acceptors of exon 7 and 9, respectively. In turn, they were predicted to cause the in-frame deletion of exon 7 (encoding 62 amino acids) and exon 9 (encoding 45 amino acids) (26,27,39,40). Both variants were predicted to cause the deletion of a significant portion of the peptide sequence in the key P450 domain. This is particularly the case in the c.802-8_810del17insGC variant, which spans the heme binding site and is critical for the protein activity.

In summary, a novel compound heterozygous mutation of c.C958T and c.G1355A in the *CYP4V2* gene was identified in a Chinese family with RP using WES. The present study not only confirmed WES to be a powerful method for screening for causative mutations for RP, but also expanded the spectrum of disease-causing variants in the *CYP4V2* gene, which will facilitate the further molecular screening of genetic variants that can cause RP.

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Availability of data and materials

The datasets used and/or analyzed during the present study are available from the corresponding author on reasonable request.

Authors' contributions

TZ, TW, FZ and SD performed the experiments and analyzed the data. BG and HZ analyzed the data and supervised the project. TZ and HZ wrote the manuscript. All authors read and approved the final manuscript. TZ, BG and HZ confirmed the authenticity of all the raw data.

Ethics approval and consent to participate

This study was approved by the Institutional Review Boards of Sichuan Provincial People's Hospital (Chengdu, China). Written informed consent was obtained from all participants or parents of children prior to their inclusion in the present study.

Patient consent for publication

Not applicable.

Competing interests

The authors declare no competing interests.

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